AN ABSTRACT OF THE THESIS OF

Shannon Elise Goff for the degree of Honors Baccalaureate of Science in Microbiology presented on June 5, 2012. Title: Strain construction to test the significance of antiactivation in *Pseudomonas* quorum sensing.

Abstract approved:

Martin Schuster

The quorum sensing regulatory pathway has been extensively studied for its impact on the virulence of *Pseudomonas aeruginosa*, an opportunistic pathogen that causes acute and chronic infections in immunocompromised individuals such as those suffering from the genetic disorder cystic fibrosis. Part of the quorum sensing pathway that has not been adequately described is the mechanism behind the accumulation of a threshold concentration of autoinducer. Recently, a protein, QteE, has been shown to inhibit the transcriptional regulator LasR. LasR binds the autoinducer molecule 3-oxo-dodecanoyl-homoserine lactone produced by the synthase LasI. QteE could also have a role in preventing the autoinducer of one cell binding its own LasR and inducing quorum sensing without a threshold concentration of autoinducer, essentially "short-circuiting" the cell. A co-culturing experiment with two QteE-deficient strains of *P. aeruginosa*, one LasI-proficient and the other LasI-deficient, would determine if this hypothesis is correct. The co-culturing experiment requires a method to differentiate between two different strains of bacteria grown together in the same medium, which in this case is tagging one strain with a red fluorescent protein, mCherry. For this thesis, I integrated a plasmid containing mCherry (pVM3) into the chromosome of the *P. aeruginosa* strains, and then confirmed their fluorescence with spectroscopy and microscopy. I also attempted to confirm the chromosomal location of the insertion by PCR.

Key words: *Pseudomonas aeruginosa*, quorum sensing, antiactivation
Corresponding email address: goffsh@onid.orst.edu

Strain construction to test the significance of antiactivation in *Pseudomonas* quorum sensing

by

Shannon Elise Goff

A PROJECT

submitted to

Oregon State University

University Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Microbiology (Honors Scholar)

Presented June 5, 2012
Commencement March 2013

Honors Baccalaureate of Science in Microbiology project of Shannon E. Goff presented on June 5, 2012.

APPROVED:

Mentor, representing Microbiology

Committee member, representing Microbiology

Committee member, representing Microbiology

Chair, Department of Microbiology

Dean, University Honors College

I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

Shannon Elise Goff, Author

ACKNOWLEDGEMENTS

I would like to thank Martin Schuster, PhD for his assistance and teaching as a mentor, as well as Rashmi Gupta for her tutelage and assistance with the lab techniques and the details of growing *P. aeruginosa*.

Additionally, I would like to thank OSU's URISC for the undergraduate research funding, and HHMI for the undergraduate research funding and program advising for presenting research in a professional manner.

Finally, I would like to thank Kevin Ahern for his part in the HHMI program advising.

TABLE OF CONTENTS

List of Tables

List of Figures

Strain construction to test the significance of antiactivation in *Pseudomonas* quorum sensing

Introduction

Pseudomonas aeruginosa

Pseudomonas aeruginosa is a Gram negative bacterium that is found ubiquitously in the environment, and is classified as a human opportunistic pathogen. Opportunists, pathogens that infect previously damaged tissue, are generally present in the environment or as commensals of their host. They are able to take advantage of locations in the host that are usually protected by the immune system, and have an innate resistance to some antibiotics [1]. *P. aeruginosa* infects individuals with weak immune systems (immunocompromised), including those with cystic fibrosis, burns, and cancer, and the case fatality rate in this group is near 50% [2].

Epidemiology and pathogenicity

In the past few decades, the population of immunocompromised individuals has increased with improved sanitation, longer lifespans and advances in medicine. The opportunities for *P. aeruginosa* to cause infection increase accordingly, through the use of indwelling catheters, ventilators, joint replacements, etc. Some quality-of-life enhancements, such as contact lenses and surgery, bypass the body's natural defenses, allowing an opportunistic pathogen entrance into protected tissues [1].

Healthcare-associated infections (HAIs) occur while a patient is being treated, in a medical environment, for another condition [3]. According to the CDC’s report of

estimated HAIs in 2002, the number of HAIs was approximately 1.7 million, and the number of deaths associated with HAIs was 98,987 [3]. *P. aeruginosa* takes advantage of those in a healthcare setting, patients who are sick, injured, burned, diseased, or otherwise immunocompromised, and can lead to HAIs. *P. aeruginosa* also has innate resistance to most commonly used antibiotics, a trait that adds to the danger of infection. Another recent CDC report on multi-drug resistant agents causing HAI ranks *P. aeruginosa* as the 6th most common agent [3]. More specifically, the bacterium is ranked second for causing ventilator-associated pneumonia, and fourth for causing catheter-associated urinary tract infections [3]. In 2002, 35,967 patients died of HAIs associated with pneumonia, and 13,088 died of HAIs associated with urinary tract infections [3].

Antibiotic resistance and biofilms

P. aeruginosa is naturally resistant to many antibiotics; however, fluoroquinolones, β-lactams, aminoglycosides and certain newer broad-spectrum antibiotics such as imipenem are still effective [1]. One mechanism that *P. aeruginosa* uses for antibiotic resistance is efflux pumps. Efflux pumps are proteins that pump an antibiotic molecule out of the cell upon entrance [1].

Another strategy *P. aeruginosa* uses for antibiotic resistance is the conversion from planktonic (or free-living) bacterial growth to biofilm growth. Bacterial biofilms are surface-attached bacterial communities encapsulated in a self-produced extracellular matrix [4]. Bacteria growing in a biofilm have increased tolerance against antibiotics and disinfectants, with a minimal bactericidal concentration of antibiotics up to 100-1000 fold higher than planktonic bacteria [4]. These biofilms can grow on the surface of catheters, implanted joints and ventilator tubes, protecting the bacteria against the immune system and antibiotics.

Quorum sensing

The infections described above involve a regulatory system known as quorum sensing (QS), which induces virulence factors [5]. The cells recognize their population density, and their behavior is altered from a harmless state to a pathogenic state. The paradigm for QS in Gram negative bacteria is based on diffusible acyl-homoserine lactone (acyl-HSL) signals, also termed autoinducers. These molecules induce gene expression in cells of the same species. The regulatory system in *P. aeruginosa* is composed of LuxI-LuxR homologs initially described in *Vibrio fischeri* [6]. In *P.aeruginosa,* two acyl-HSL signaling systems, LasI-LasR and RhlI-RhlR, exist [7]. In the following, the LasI-LasR system will be described in more detail as the major system which also controls the activity of the subordinate RhlI-RhlR system [7]. The acyl-HSL autoinducer, 3-oxo-dodecanoyl (3OC12)-HSL, is produced by the LasI autoinducer synthase protein. The autoinducer leaves the cell and diffuses into the media. As more cells accumulate, the autoinducer concentration increases, indicative of cell density. When the autoinducer reaches a threshold concentration and binds to the LasR transcriptional regulator, the combined autoinducer and LasR complex induce transcription of QS regulated genes. The LasR-autoinducer complex also binds to the promoter region of the *lasI* gene, further increasing autoinducer synthesis.

Many important extracellular virulence factors are controlled by QS, such as the exoprotease elastase (LasB) and *Pseudomonas* toxin (ExoA), which is cytotoxic to epithelial cells [8]. Also, QS is involved in biofilm formation. Biofilms produced by bacteria lacking QS are structurally different from those formed by the wild-type [9]. Therefore, QS is important in the ability of this bacterium to cause disease, and QS in *P. aeruginosa* has been proposed as a target for alternative drugs. Unlike traditional antibiotics, antivirulence

drugs attack the virulence of a bacterium rather than a mechanism required for viability [10]. Shutting off all the QS-controlled genes in *P. aeruginosa* would greatly reduce the ability to cause infection. The most beneficial aspect of antivirulence drugs is that they do not impose harsh selective pressure.

QteE

Within QS, how is the characteristic induction threshold achieved? How does the autoinducer avoid the free LuxR transcription factor in the cytoplasm of the cells? Theoretically, after LuxI produces the autoinducer, the molecule diffuses out of the cell and is prevented from binding the LuxR transcription regulator until the threshold concentration is reached. What prevents the autoinducer from binding LuxR before leaving the cell, or from binding any LuxR the autoinducer contacts before the threshold is reached? The solution to this enigma is thought to be a process termed antiactivation. An antiactivator protein suppresses the ability of LuxR to bind to the autoinducer. The antiactivator was first described in the QS system of *Agrobacterium tumefaciens* as the protein TraM. TraM suppresses the activation of QS controlled genes by direct interaction with TraR. Deletion of the *traM* gene induced constitutive transcription of QS-controlled genes [11,12]. In 2010, an analog to TraM was found in the *P. aeruginosa* QS system. QteE (quorum threshold expression element) was found to reduce the stability of LasR, presumably through direct protein-protein interaction [13]. Experiments showed that over-expression of LasR overcame QteE's effects and that *lasR* expression increased in exponential phase [13], leading to the theory that a threshold is reached when the concentration of LasR in the cell is greater than the concentration of QteE. Computational modeling of the *A. tumefaciens* QS pathway suggested that the TraR-TraM negative feedback loop is essential to prevent short-circuiting [14].

Objective of research

Here, we describe preliminary experiments that enable experimental testing of the prediction that the antiactivator QteE functions to prevent short-circuiting in *P. aeruginosa* QS. Without an antiactivator, the autoinducer may bind LasR directly after being synthesized, in the same cell. The LasR-autoinducer complex would then initiate QS in its own cell (short-circuiting), which defeats the purpose of the QS pathway.

The long-term goal is to fully understand the design principles of QS signaling in bacteria like *P. aeruginosa*. Identifying all the parts of the QS pathway, and understanding their functions, increases the success of using QS as a target for antivirulence drugs. These drugs need a well-characterized target, and targets that are specific to a pathogenic bacterium will increase the specificity of the drug, which is a highly valued attribute.

The experimental approach is to construct strains lacking QteE and to monitor the induction of a QS target gene in co-culture by fluorescence microscopy. One of the two *P. aeruginosa* strains to be used in co-culture is a *qteE* mutant (PAO1-SG3), and the other strain is a *qteE lasI* double mutant, PAO1-SG4 (Table 1). The two strains are differentiated in co-culture by chromosomally labeling one strain with the mCherry gene, encoding a red fluorescent protein. QS induction is measured using a green fluorescent protein (GFP) reporter. The autoinducer is synthesized in PAO1-SG3, and the local concentration of the autoinducer is visualized by GFP induction. If short-circuiting occurs when a cell lacks QteE, then PA01-SG3 will induce GFP before PA01-SG4 does, following the diffusion of the autoinducer (Figure 1, E-H). In contrast, control strains expressing QteE, the PAO1 wild-type (WT), PAO-SG, and an isogenic *lasI* mutant (PAO-SG2) are expected to induce GFP at the same time in co-culture (Figure 1, A-D).

Table 1. Genotype and function of the four strains used in this thesis.

Name	Genotype	Function
PAO-SG1	Wild-type	QS functions as normal (control)
PAO-SG2	*lasI*$^-$	*lasI* deletion. Autoinducer will not be synthesized (control)
PAO-SG3	*qteE*$^-$	*qteE* deletion. LasR will be available to bind to any available autoinducer
PAO-SG4	*lasI qteE*$^-$	*lasI* and *qteE* deletion. LasR will be available to bind to any autoinducer, and the cell cannot produce its own autoinducer

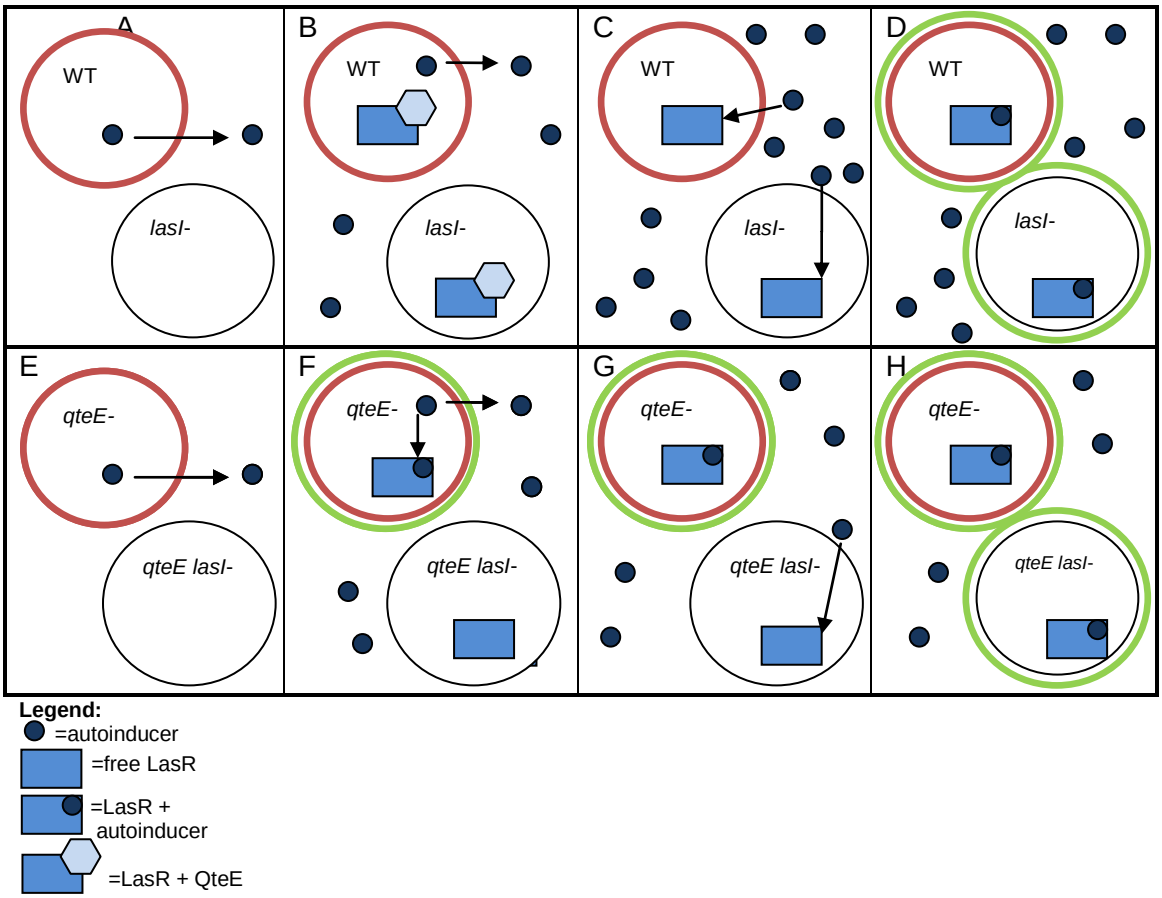

Figure 1. Model depicting the predicted temporal sequence of interactions between *P. aeruginosa* strains in co-culture. A-D: Control experiment with the WT and the *lasI* mutant exhibiting predictable behavior. A) The WT (red) will produce the autoinducer, which will diffuse out of the cell. *lasI*- will not produce an autoinducer. B) QteE reduces the half-life of LasR, allowing the concentration of the autoinducer to increase, until the threshold has been reached. C) When the threshold has been reached, the LasR concentration will overcome the QteE concentration. D) Both cells will fluoresce green at the same time. E-H: Experiment with *qteE* and *lasI qteE* mutants. Neither cell can express QteE. E) *qteE*- (red) will produce autoinducer, *qteE lasI*- will not produce an autoinducer. F) The autoinducer will diffuse out of the cell, or can come into contact with its own LasR. The LasR-autoinducer complex will induce QS and initiate green fluorescence. G) Eventually, the autoinducer will come into contact with the *qteE lasI*- LasR. H) The LasR-autoinducer complex will induce QS, and the cell will fluoresce green.

The experiment will be completed in three parts. First, the strains are tagged with red fluorescence to be used for differentiation in co-culture. We used pVM3, a miniCTX plasmid which includes a gene for mCherry, a red fluorescent protein. The plasmid is non-replicating in *P. aeruginosa*, and can be transferred from *E. coli* through transformation or electroporation [21]. The miniCTX plasmid contains a ϕCTX attachment site and the *int* gene, allowing the integration of the plasmid at the specific *attB* site in *P. aeruginosa*. We measured mCherry fluorescence in the constructed strains using fluorescence microscopy and a fluorescence plate reader. We also attempted to confirm the position of the mCherry integration using polymerase chain reaction (PCR). The miniCTX plasmid is designed to integrate at the *attB* site, and can be confirmed using primers that flank the *attB* site [21]. Second, the strains are tagged with green fluorescent protein (GFP) on a plasmid. The plasmid contains the GFP gene controlled by the QS-controlled LacZ promoter. The last step is running the co-culturing experiment. The strains will be grown to the exponential growth stage, then diluted to a low density. The fluorescence will be monitored by microscopy. In this thesis, we describe the first step, namely the construction and validation of mCherry-labeled *P. aeruginosa* strains.

Materials and Methods

Strains and plasmids

Four strains of *P. aeruginosa* were used in this project: PAO1 wild-type (WT), *lasI* mutant, *qteE* mutant, and *lasI qteE* double mutant. During the course of this project, each of these strains was tagged with mCherry (red fluorescent protein derivative). All strains and plasmids used are listed in Table 2.

Growth Conditions

P. aeruginosa and *E. coli* were grown in Lennox Lysogeny Broth (LB) or Heart Infusion (HI) medium at 37°C. Antibiotics were used in selection media at the following concentrations: tetracycline (*P. aeruginosa*, 100 µg ml^{-1}), gentamicin (*E. coli,* 10 µg ml^{-1} *P. aeruginosa,* 100 µg ml^{-1}), ampicillin (*E. coli*, 100 µg ml^{-1}), and carbenicillin (*P. aeruginosa*, 200-300 µg ml^{-1}).

Introduction of plasmids by transformation

Transformation (the uptake of foreign DNA by a competent cell) of *E. coli* was performed using the polyethylene glycol method [19]. First, competent cells were prepared. An *E. coli* SM10 overnight culture was diluted to an optical density (OD_{600}) of 0.05 and grown to mid-exponential phase (OD_{600} of 0.8). One ml of culture was pelleted in the centrifuge (max. speed for 30 s). The supernatant was discarded, and the residual LB (50 µL) was used to re-suspend the cell pellet. One hundred µL of transformation buffer was added to each tube. Transformation buffer contained LB broth, 10% (wt/vol) polyethylene glycol, 5% (vol/vol) dimethyl sulfoxide, and 50 mM Mg^{2+} ($MgSO_4$ or $MgCl_2$) at pH 6.5 [20]. Then

Table 2. Strains and plasmids.

Strain or plasmid	Relevant property	Reference
Strain		
Pseudomonas aeruginosa		
PAO1	Wild-type (Ochsner lineage)	[15]
PAO-SG2	*lasI* deletion strain (Ochsner)	[16]
PAO-SG3	transposon insertion mutation *qteE::*IS*lacZ*/hah (Ochsner), introduced from PAO1 (UW) library strain	Verena Mueller, Schuster Lab, unpublished
PAO-SG4	*lasI* deletion strain with *qteE* mutation (Ochsner), double mutant	Verena Mueller, Schuster Lab, unpublished
PAO-SG1mc	mCherry (pVM3) integrated into wild-type	This study
PAO-SG2mc	mCherry (pVM3) integrated into *lasI* deletion strain	This study
PAO-SG3mc	mCherry (pVM3) integrated into *qteE* mutant	This study
PAO-SG4mc	mCherry (pVM3) integrated into double mutant	This study
PAO-SG1mcx	mCherry (pVM3) integrated into wild-type, plasmid backbone excised	This study
PAO-SG2mcx	mCherry (pVM3) integrated into *lasI* deletion strain, plasmid backbone excised	This study
PAO-SG3mcx	mCherry (pVM3) integrated into *qteE* mutant, plasmid backbone excised	This study
PAO-SG4mcx	mCherry (pVM3) integrated into double mutant, plasmid backbone excised	This study
Escherichia coli		
SM10	Conjugation-proficient strain. Km^R, *thi-1, thr, leu, tonA lacY, supE, recA::RP4-2-Tc::Mu, pir*	Lab collection
DH5a	General cloning host. F-ϕ80*lacZ* ΔM15 Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR17* (r_k^- m_k^+) *phoA supE44 thi-1 gyrA96 relA1* λ-	[17], Invitrogen
Plasmids		
pVM3	Integration-proficient mobilizable broad-host range vector miniCTX3a, constitutively expressing mCherry	Verena Mueller, Schuster Lab, unpublished
pFLP2	Source of Flp recombinase	[18]

600-800 µg of purified plasmid DNA was added to the competent cells. The tubes were all incubated on ice for 20 minutes and heat-shocked at 37°C for 1.5 minutes. The cells

were cooled on ice again for 3 minutes, suspended in 950 µL LB media and allowed to recover at 37°C, with shaking, for 1 hour. Finally, 100 µL of each culture was plated on LB plates containing gentamicin. The remaining volume was concentrated and also plated. The plates were incubated overnight at 37°C. The gentamicin-resistant colonies were streaked for isolation and prepared as a freezer stock. A negative control consisted of competent cells without DNA.

Conjugation to construct chromosomal mCherry strains

Conjugation is the transfer of plasmid from one bacterial strain to another. We used the conjugation-proficient *E. coli* SM10 strain to insert pVM3 into *P. aeruginosa* strains. The donor strain, *E. coli* SM10/pVM3, and the recipient strains, *P. aeruginosa* WT and mutants, were grown overnight on selective media plates. Bacterial colonies were scraped from the plate and suspended in 1 ml of HI broth, after which the OD_{600} was measured. An aliquot of this suspension was diluted in 4 ml of LB broth to a calculated OD_{600} of 0.2 (donor strain with plasmid) or 0.05 (recipient strains). The donor strain required antibiotic media to continue replication of the plasmid; therefore, the donor strain grew more slowly and was started at a higher concentration. The cultures were grown to mid-exponential phase, an OD_{600} of approximately 0.6. For mating, the cells should be at a 1:1 ratio. Using the OD_{600} measurements as an estimate for cell concentration, the appropriate volumes were calculated. The calculated aliquots were then placed into separate microcentrifuge tubes, one for the recipient and one for the donor. Cells were washed in 1 ml of phosphate-buffered saline and re-suspended in 25 µL HI broth. The donor cells and recipient cells were mixed, resulting in a total volume of 50 µL. The mixture was spotted on LB plates and grown overnight. The next morning, the spot was scraped off the plate and suspended in 1 ml of HI broth. The tube was thoroughly mixed,

suspending all clumps of cells. The entire mixture was plated in 200 µL aliquots on *Pseudomonas* Isolation Agar containing 100 µg ml^{-1} gentamicin. Gentamicin-resistant *P. aeruginosa* colonies grew overnight and indicated an integrated plasmid, as pVM3 cannot replicate in *P. aeruginosa*. The colonies were streaked for isolation and prepared as freezer stock.

Electroporation to construct chromosomal mCherry strains

The process of electrically shocking cells to become competent for the intake of foreign DNA is termed electroporation. This is another method used to insert the pVM3 plasmid into *P. aeruginosa* strains. We used electroporation after the initial attempts with conjugation were unsuccessful. The recipient strains were inoculated in two tubes of 4 ml LB broth (total of 8 ml culture) and grown overnight at 37°C with shaking. Then, 1.5 ml of culture was distributed into 4 microcentrifuge tubes and centrifuged at 16,000xg for two minutes. Cells were pelleted and re-suspended in 1 ml of 300 mM sucrose. The cells were washed with sucrose twice and pelleted. The four washed pellets of cells were suspended in a combined volume of 200 µL of 300 mM sucrose. Half of this volume was transferred to a 2 mm gap-width electroporation cuvette, and 1-3 µL of DNA was added and mixed by stirring. The other half was also added to a cuvette, but no DNA was added. This served as a negative control. Electroporation was accomplished under these conditions: 25 µF, 200 Ω, 2.5 kV. Immediately after shocking, 1 ml LB for the negative control or 1 ml SOC (Super Optimal Broth with Catabolite Repression) for the test culture was added for recovery. One hundred µL of culture was then plated on LB containing gentamicin. The remaining culture was concentrated by centrifugation, re-suspended in 100 µL LB or SOC and also plated. After overnight incubation, any colonies that grew

were gentamicin resistant and indicated an integrated plasmid. The gentamicin-resistant colonies were streaked for isolation and prepared as a freezer stock.

PCR confirmation of pVM3 integration

We used polymerase chain reaction (PCR) to determine if the mCherry gene integrated into the *P. aeruginosa* chromosome at the predicted site. pVM3 is designed to integrate at a ϕCTX phage site using *int* genes included in the plasmid [21]. DNA was isolated using the Gentra Puregene Yeast/Bact Kit B. The PCR reaction mixture and PCR program are detailed in Tables 3 and 4. The forward and reverse primers are 5'-TGC GAA TGA CCT TGA GTT TG-3' and 5'-GAC CTC TAG GGT CCC CAA TTA-3', respectively [21]. After the PCR reaction was complete, each reaction mixture was resolved on a 0.7% agarose gel run at a 90V.

Table 3. PCR reaction mixture.

Ingredient	Amount
Template	600 ng
30 µg µl^{-1} Forward primer	1 µl
30 µg µl^{-1} Reverse primer	1 µl
250U Taq polymerase	0.7 µl
10 mM dNTPs	2.5 µl
10x Buffer 3	5 µl
H_2O	ad 30 µl

Table 4. PCR program.

Step	Temperature	Time	Cycles
Precycle	95°C	2 minutes	1
Denaturation	95°C	30 seconds	30
Annealing	60°C	30 seconds	
Extension	68°C	1 to 6 minutes[1]	
Final extension	68°C	10 minutes	1
Cooling	4°C	-	1

[1] extension time varied with length of amplicon

pFLP2 excision of pVM3 plasmid backbone

To reduce the size of the mCherry insert (pVM3) from 6.3 kb to 2.8 kb [20, 21], we excised the backbone of the chromosomally situated vector using Flp recombinase carried on the plasmid pFLP2 [18]. Competent cells containing the integrated plasmid were transformed with pFLP2 and then plated on LB containing carbenicillin. The carbenicillin-resistant transformants were screened for loss of the plasmid backbone due to Flp recombinase by patching on two different LB plates: one with carbenicillin and the other with gentamicin. The transformants that grew on carbenicillin plates but not on gentamicin plates were picked and patched on LB plates containing 5% sucrose. These colonies were selected for the loss of pFLP2 by patching on two different plates: LB with carbenicillin and LB plates containing 5% sucrose. Those that grew on LB plates containing 5% sucrose and did not grow on carbenicillin plates were streaked on LB plates for isolation and prepared for freezer stocks, as excised integrants.

Fluorescence measurement with multi-function plate reader

The instrument used for measuring fluorescence was a multi-function plate reader (Infinite M200, Tecan), which elicits and detects a fluorescence response in a culture. For mCherry, the excitation wavelength is 587 nm and the emission wavelength is 610 nm. Each culture sample was measured at an optical density (OD_{600}) of 0.5. In taking the measurements, the background fluorescence of the dilution buffer was subtracted from the fluorescence of the samples.

Fluorescence microscopy

Microscopy was used to detect individual fluorescent cells. Two modes, differential interference contrast (DIC) and fluorescence, were used. DIC is a setting used to increase contrast in unstained, transparent samples using complex lighting patterns. Fluorescence microscopy uses a mercury halogen lamp and appropriate filters to produce fluorescence at wavelengths specific to mCherry and other fluorescent proteins. Microscope slides were prepared using thin 1% agarose gel slices (0.25 g agarose, 25 ml PBS) prepared in an SDS-page gel casting apparatus. Samples were prepared from overnight cultures. The cells were washed in PBS and diluted to an OD_{600} of 0.5. A two-by-two cm square was cut from the prepared gel and placed on the microscopy slide. Bacteria were added in a 10 µl drop and allowed to dry. A drop of silicon at each corner held a coverslip in place over the agarose gel square. The use of agarose pads restricted the movement of the bacteria for improved image quality.

We used a Leica microscope, with the Leica DC500 camera, for both DIC and fluorescence settings. The images were all taken using the 63x objective and immersion oil.

Results

Confirmation of the chromosomal integration of mCherry by PCR

The mCherry construct, carried on an integration-proficient plasmid, pVM3, was integrated into the chromosome of *P. aeruginosa* WT and mutant strains as described in *Materials and Methods*. The plasmid contains a phage (ϕCTX) attachment site, permitting integration into a corresponding *attB* site in the *P. aeruginosa* chromosome using integrase-mediated recombination [21]. To locate the insert in the chromosome, we used primers that flank the phage insertion site and amplify that region by PCR. An amplicon of 270 base pairs corresponds to the ϕCTX *attB* site without an insert, and an amplicon of 6.3 kb corresponds to the construct containing the pVM3 insert [18]. Figure 2 depicts a preliminary PCR run with the two strains that had mCherry integrated in the chromosome, as the other two strains were not yet available. The WT negative control (lane 2) has a band at 270 bp, as desired. The putative WT-mCherry and *qteE lasI*-mCherry strains (lanes 3-6) have a less intense band of identical size. The entire plasmid construct, if integrated at the *attB* site, would have resulted in a PCR product of 6.3 kb. However, we did not observe a band of the corresponding size. To exclude the possibility that the lack of a 6.3 kb amplicon is not due to the relatively short PCR extension time of 3 min., we conducted another PCR in which we lengthened the extension time to 6 min. This PCR also did not amplify a band of the desired length. These preliminary results indicate that the plasmid did not integrate at the *attB* site. We then excised the integrated plasmid backbone with Flp recombinase to reduce the construct to a smaller 2.8 kb. The PCR amplification with the smaller 2.8 kb insert has a higher prevalence for resulting in a complete amplicon than the longer 6.3 kb insert. However, a PCR after excision (Figure 3) also did not yield a band of the appropriate size, and there were no visible bands

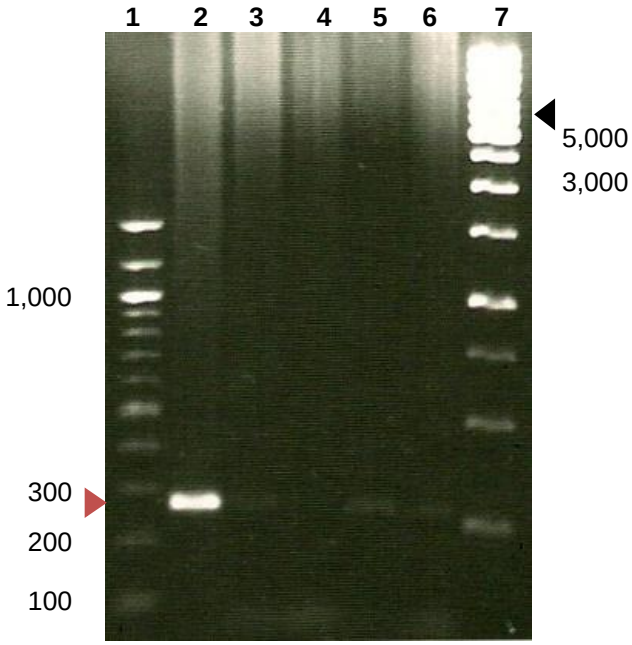

Figure 2. Initial PCR of mCherry strains. Lane 1, 100 bp ladder; lane 2, WT negative control; lanes 3-5, three WT-mCherry candidates; lane 6, *qteE lasI*-mCherry candidate; lane 7, 1 kb ladder. The band at 270 bp (red arrow head) indicates an amplicon without any insertion. The desired band for the samples in lanes 3-6 would be at 6.3 kb (black arrow head). Lanes 3, 5, and 6 show a faint band at 270 bp (red arrow head).

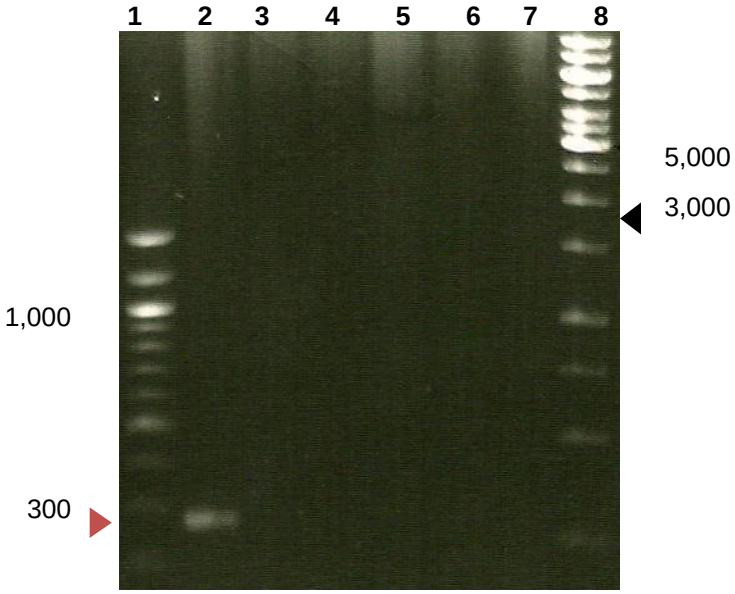

Figure 3. PCR of excised mCherry strains. Lane 1, 100 bp ladder lane 2, WT negative control; lane 3, excised WT-mCherry candidate; lane 4, excised *lasI*-mCherry candidate; lane 5, excised *qteE*-mCherry candidate; lane 6, excised *qteE lasI*-mCherry candidate; lane 7, miniCTX-*lacZ* insert positive control, 4.6 kb (Schuster lab); lane 8, 1 kb ladder;. Lane 2 has a band at 270 bp (red arrow head), indicative of the amplicon obtained with no insert. The desired size of the amplicon with the insert would be 2.8 kb (black arrow head), and is not visible.

matching the negative control, although the exposure time of the gel image is shorter than that in Figure 2. These results were reproducible even after checking for contamination of the DNA samples.

Confirmation of mCherry fluorescence by fluorescence spectroscopy

Despite the negative PCR results, fluorescence was confirmed in all of the mCherry-tagged strains (Figure 4). Strains were grown in culture, then measured in a multifunction plate reader for red fluorescence. Both the integrants with the whole plasmid and the integrants with the excised plasmid measured significant fluorescence in comparison with the mutant strains that were not exposed to the plasmid (Figure 4).

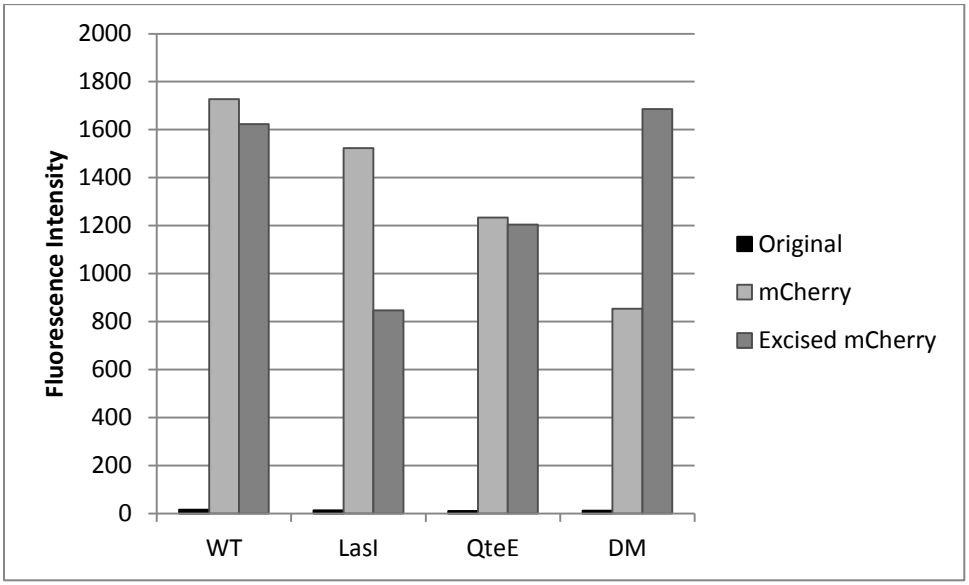

Figure 4. mCherry fluorescence measured with multi-function plate reader. For each of the four bacterial strains, three measurements were taken: the strain before pVM3 integration (without mCherry, black); the strain right after pVM3 integration (with mCherry, light grey); and the strain with excised pVM3 (with mCherry, excised, dark grey).

Confirmation of mCherry fluorescence by microscopy

In the planned co-culture experiment, cellular fluorescence will be observed using fluorescence microscopy. Therefore, ensuring that mCherry functions in every individual cell of the tagged strains is essential. To verify that each cell fluoresces, a field of view was captured in DIC mode first. DIC enhances the contrast for optimal visualization of an unstained specimen. The same field of view was then captured in fluorescence mode (Figure 5). We confirmed the fluorescence of 100% of the cells in all four mutant strains, both excised and not excised, using this technique.

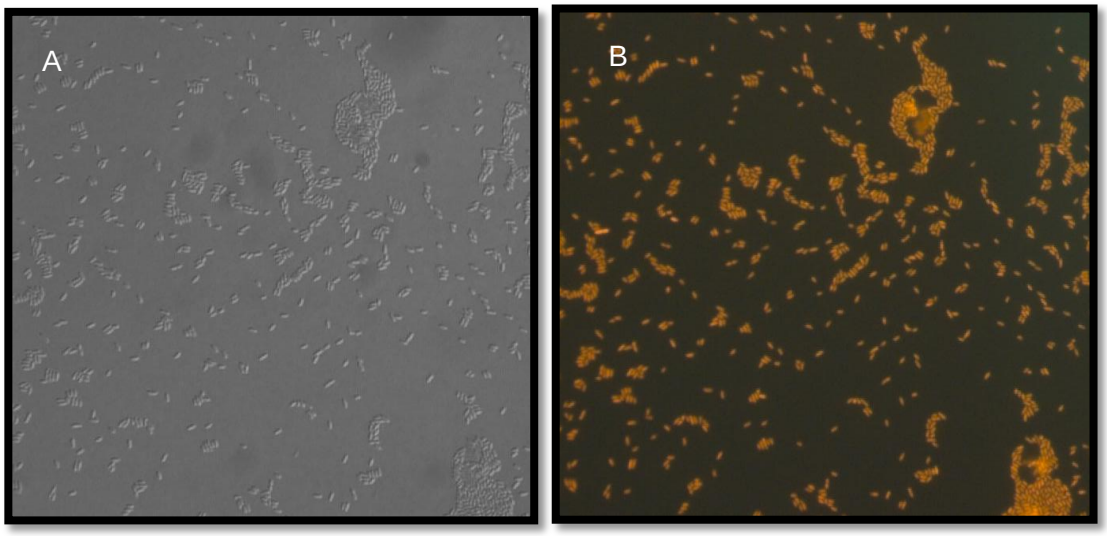

Figure 5. DIC and fluorescence microscopy images. The WT-mCherry strain was magnified with the 63x objective. A) DIC. B) Same field of view by fluorescence microscopy.

Discussion

To differentiate between two strains of *P. aeruginosa* in a co-culturing experiment, we integrated a plasmid containing the gene for mCherry into the chromosome of four different *P. aeruginosa* strains. We characterized the resulting strains by PCR, fluorescence spectroscopy and fluorescence microscopy. Both spectroscopy and microscopy proved that mCherry is expressed by each strain.

The fluorescence of the four strains was confirmed using fluorescence microscopy and fluorescence spectroscopy; however, the integration site was not confirmed using PCR. PCR amplification of DNA from the mCherry-tagged strains did not show a band at the appropriate size, but instead showed a smaller, faint band matching that of the negative control. Even after FLP-recombinase excision of the plasmid backbone, PCR did not reveal a band of the expected size of 2.8 kb. These results indicate that the amplicon did not insert in the desired position. We know the primers to be correct, as the negative control worked properly. Before moving on to the next step in the experiment, we need to confirm the placement of the mCherry insert. If the plasmid inserted elsewhere, it could have inserted in the middle of a gene and impair some function in the bacteria, skewing any data gained by using the strain.

It would be prudent to repeat the PCR with a positive control, a strain with the same integrated plasmid that has had positive PCR results in the past. The chosen amplification conditions should yield an band of the appropriate size. If the PCR continues to be inconclusive, the next step would be to verify that the mCherry gene is present in the chromosome, using primers internal to the mCherry coding region. If these parameters indicate the presence of the mCherry gene, then the amplicon likely inserted incorrectly.

The insertion location could be determined by arbitrary PCR. In this method, one primer is sequence-specific, targeting the known insertion sequence, and the other primer is of semi-random sequence composition, permitting amplification and subsequent sequencing of the region adjacent to the insert. Locating the mCherry insert could allow use of the strain, if it is not found to be disrupting any important function.

Once correct strain construction has been verified, the next step in the co-culturing experiment will be the introduction of a plasmid containing the green fluorescent protein (GFP) gene under the control of a strong QS promoter. The plasmid will act as an indicator of QS activation. The last step of the experiment will be the co-culturing with fluorescence microscopy as shown in Figure 1.

The importance of this work is in understanding the design principles of a QS network for synthetic biology applications, and in understanding the mechanisms that control virulence gene expression in the bacterial pathogen *P. aeruginosa*. In understanding all of the pieces of QS, and how this regulatory system is involved in causing infection, the process of creating successful antivirulence drugs is possible.

Literature cited

1. **Wilson BA, Salyers AA, Whitt DD, Winkler ME**. 2002. *Bacterial Pathogenesis: A Molecular Approach.* 3rd ed. Washington, DC: ASM Press.

2. **EHA Consulting Group.** 2012. What is *Pseudomonas aeruginosa*? [Online] http://www.ehagroup.com/resources/pathogens/pseudomonas-aeruginosa/

3. **Hidron AI, Edwards JR, Patel J, Horan TC, Sievert MD, Pollock DA, Fridkin SK.** 2008. Antimicrobial-Resistant Pathogens Associated With Healthcare-Associated Infections: Annual Summary of Data Reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2006–2007. Infect. Control Hosp. Epidemiol. **29**:996-1011.

4. **Hoiby N, Ciofu O, Johansen HK, Song ZJ, Moser C, Jensen PO, Molin S, Givzkov M, Tolker-Nielsen T, Bjarnsholt T.** 2011. The clinical impact of bacterial biofilms. Int. J. Oral. Sci. **3**:55-65.

5. **Popat R, Crusz SA, Diggle SP.** 2008. The social behaviours of bacterial pathogens. Br. Med. J. **87**:63-75.

6. **Nealson KH, Platt T, Hastings JW.** 1970. Cellular Control of the Synthesis and Activity of the Bacterial Luminescent System. J. Bacteriol. **104**:313-322.

7. **Schuster M, Greenberg EP**. 2006. A network of networks: quorum-sensing gene regulation in *Pseudomonas aeruginosa.* Int. J. Med. Microbiol. **296**:73-81.

8. **Schuster M, Lostroh CP, Ogi T, Greenberg EP.** 2003. Identification, Timing and Signal Specificity of *Pseudomonas aeruginosa* Quorum-Controlled Genes: a Transcriptome Analysis. J. Bacteriol. **185**:2066-2079

9. **Davies DG, Parsek MR, Pearson JP, Iglewski BH, Costerton JW, Greenberg EP.** 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science. **280**:295-8.

10. **Rasko DA, Sperandio V**. 2010. Anti-virulence strategies to combat bacteria-mediated disease. Nat. Rev. Drug Discov. **9**:117-28.

11. **Hwang I, Cook DM, Farrand SK.** 1995. A new regulatory element modulates homoserine lactone-mediated autoinduction of Ti plasmid conjugal transfer. J. Bacteriol. **177**:449.

12. **Fuqua C, Burbea M, Winans SC.** 1995. Activity of the *Agrobacterium* Ti Plasmid Conjugal Transfer Regulator TraR is Inhibited by the Product of the *traM* Gene. J. Bacteriol. **177**:1367-1373.

13. **Siehnel R, Traxler B, An DD, Parsek MR, Schaefer AL, Singh PK.** 2010. A unique regulator controls the activation threshold of quorum-regulated genes in *Pseudomonas aeruginosa*. PNAS. **207**:7916-21.

14. **Goryachev AB.** 2011. Understanding Bacterial Cell-Cell Communication with Computational Modeling. Chem. Rev. **111**:238-250.

15. **Rahim R, Ochsner UA, Olvera C, Graninger M, Messner P, Lam JS, Soberon-Chavez G.** 2001. Cloning and functional characterization of the *Pseudomonas aeruginosa rhlC* gene that encodes rhamnosyltransferase 2, an enzyme responsible for di-rhamnolipid biosynthesis. Mol. Microbiol. **40**:708-18.

16. **Wilder CN, Diggle SP, Schuster M**. 2011. Cooperation and cheating in *Pseudomonas aeruginosa*: the roles of the *las, rhl* and *pqs* quorum-sensing systems. ISME J. **5**1332-43.

17. **Hanahan D**. 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166**:557-80.

18. **Hoang TT, Karkhoff-Schweizer RR, Kutchma AJ, Schweizer HP**. 1998. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. Gene. **212**:77-86.

19. **Klebe RJ, Harriss JV, Sharp DZ, Douglas MG**. 1983. A general method for polyethylene-glycol-induced genetic transformation of bacteria and yeast. Gene. **25**:333-41.

20. **Chung CT, Niemela SL, Miller RH**. 1989. One-step preparation of competent *Escherichia coli*: transformation and storage of bacterial cells in the same solution. PNAS. **86**:172-75.

21. **Hoang TT, Kutchma AJ, Becher A, Schweizer HP**. 2000. Integration-proficient plasmid for *Pseudomonas aeruginosa*: site-specific integration and use for engineering of reporter and expression strains. Plasmid. **43**:59-72.